LIGNOCELLULOLYTIC ENZYMES BY *Aspergillus* sp. A1 AND *Bacillus* sp. B1 ISOLATED FROM GUT OF *Bulbitermes* sp. IN SOLID STATE FERMENTATION USING SAWDUST AS SUBSTRATE

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Specially dedicated to supportive families and friends who had been an inspiration to me to be a better person

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ABSTRACT

Sawdust is one of the common lignocellulosic waste biomass produced during the process of planning mills, moulding plants and furniture manufacturing. In practice, the sawdust is discarded in landfill areas, causing dust and dirt pollution in nearby localities. Therefore, the need to find an efficient and practical approach to revalorize sawdust as a starting raw material in the production of lignocellulolytic enzymes is essential as a way to manage and turn the residues into value added products. Prospecting for efficient degrading lignocellulose microorganisms is crucial to facilitate the process of lignocellulolytic enzymes production from the lignocellulosic biomass. This study aimed to exploit microorganisms isolated from gut of termite *Bulbitermes* sp. in producing lignocellulolytic enzymes under solid-state fermentation (SSF) system by using untreated sawdust as substrate. Seventeen bacterial and five fungal with positive lignocellulolytic enzymes activities were successfully isolated from the gut of two hundred termites. Four isolates identified as *Aspergillus* sp. A1, *Bacillus* sp. B1, *Bacillus* sp. B2 and *Brevibacillus* sp. Br3 were selected for further characterization. Among the isolates, *Aspergillus* sp. A1 showed highest activities of lignin peroxidase (LiP) (729.12 U/g) and β-glucosidase (22.97 U/g). The highest activities of endoglucanase (138.77 U/g) and manganese peroxidase (MnP) (47.73 U/g) were recorded in *Bacillus* sp. B1. The *Bacillus* sp. B2 produced the highest activities of exoglucanase (32.16 U/g) and laccase (71.18 U/g). The highest xylanase activity (104.96 U/g) was observed in *Brevibacillus* sp. Br3. The production of endoglucanase, β-glucosidase, xylanase, LiP and laccase were approximated 17–93% higher in co-culture compared to individual culture. Compared to other di-, tri- and quad-mixed culture, *Aspergillus* sp. A1 (A1) and *Bacillus* sp. B1 (B1) co-culture produced the highest lignocellulolytic enzymes activities (endoglucanase, 190.1; exoglucanase, 13.5; β-glucosidase, 33.7; xylanase, 202.5; LiP, 713.5; MnP, 23.3 and laccase, 52.1 U/g). The interaction between A1 and B1 is not antagonistic. Study on the effect of SSF operational variables showed that the use of unsieved sawdust produced significantly higher activities of exoglucanase, xylanase, LiP and laccase compared to that of sieved sawdust. In addition, temperature, pH and moisture content significantly impacted lignocellulolytic enzymes production. In comparing to control, moistening the unsieved sawdust with Mandel basal medium (pH 8) to 1:2.5 (solid:moisture) ratio, and incubation at 35 °C for 9 days produced 1.2–49.4 fold higher lignocellulolytic enzymes activities. Endoglucanase, β-glucosidase and xylanase could be classified as moderately thermostable enzymes with better stability in acidic pH range. Meanwhile, ligninases possessed thermophilic and alkaliphilic characteristics. The co-culture produced 1.9–11.8 fold higher reducing sugars than those yielded by single cultures in the enzymatic degradation of sawdust. The use of co-culture enzymes also produced 3.6–85.4% higher reducing sugars as well as 1.3–2.3 times higher raffinose, cellobiose, maltose, glucose and xylose concentrations compared to that of commercial cellulase (Celluclast) solution. As conclusion, this work has generated a microbial co-culture that could be used for improved lignocellulolytic enzymes and reducing sugars production using untreated sawdust as substrate.

ABSTRAK

Hampas kayu merupakan salah satu sisa biojisim lignoselulosa yang dihasilkan semasa proses pengilangan terancang, loji pengacuan dan pembuatan perabot. Kebiasaannya, hampas kayu dibuang di kawasan pelupusan sampah, mengakibatkan pencemaran habuk dan debu di kawasan setempat yang berhampiran. Oleh itu, mencari pendekatan yang efisien dan praktikal untuk meningkatkan nilai hampas kayu adalah penting sebagai cara untuk mengurus dan menukar sisa ini kepada produk berguna dengan menggunakannya sebagai bahan asas dalam penghasilan enzim lignoselulolitik. Pengenalpastiaan mikroorganisma yang boleh menguraikan lignoselulosa secara efisien adalah penting untuk memudahkan proses penghasilan enzim lignoselulolitik dari biojisim lignoselulosik. Matlamat kajian ini adalah untuk mengeksploitasi mikroorganisma yang dipencilkan daripada usus anai-anai *Bulbitermes* sp. dalam menghasilkan enzim lignoselulolitik di bawah sistem penapaian keadaan pepejal (SSF) menggunakan hampas kayu yang tidak dirawat sebagai substrat. Tujuh belas bakteria dan lima kulat dengan aktiviti enzim lignoselulolitik yang positif telah berjaya dipencilkan daripada usus dua ratus anai-anai. Empat pencilan dikenalpasti sebagai *Aspergillus* sp. A1, *Bacillus* sp. B1, *Bacillus* sp. B2 dan *Brevibacillus* sp. Br3 telah dipilih untuk pencirian yang lebih lanjut. Diantara pencilan-pencilan tersebut, *Aspergillus* sp. A1 menunjukkan aktiviti lignin peroksidase (LiP) (729.12 U/g) dan β-glukosidase tertinggi (22.97 U/g). Aktiviti endoglukanase (138.77 U/g) dan manganese peroksidase (MnP) (47.73 U/g) tertinggi telah direkodkan oleh *Bacillus* sp. B1. *Bacillus* sp. B2 menghasilkan aktiviti eksoglukanase (32.16 U/g) dan lakase (71.18 U/g) tertinggi. Aktiviti xilanase tertinggi (104.96 U/g) dicatatkan oleh *Brevibacillus* sp. Br3. Penghasilan endoglukanase, β-glukosidase, xilanase, LiP dan lakase adalah dianggarkan 17–93% lebih tinggi dalam kultur bersama berbanding dengan kultur tunggal. Perbandingan antara dwi-, tri- dan kuad-kultur bercampur menunjukkan, kultur bersama *Aspergillus* sp. A1 (A1) dan *Bacillus* sp. B1 (B1) menghasilkan aktiviti enzim lignoselulolitik tertinggi (endoglukanase, 190.1; eksoglukanase, 13.5; β-glukosidase, 33.7; xilanase, 202.5; LiP, 713.5; MnP, 23.3 dan lakase, 52.1 U/g). A1 dan B1 mempunyai hubungan tidak antagonis. Kajian mengenai kesan parameter operasi SSF menunjukkan hampas kayu yang tidak diayak menghasilkan aktiviti eksoglukanase, xilanase, LiP dan lakase jauh lebih tinggi berbanding dengan hampas kayu yang diayak. Selain itu, suhu, pH dan kandungan kelembapan memberi kesan yang signifikan terhadap penghasilan enzim lignoselulolitik. Hampas kayu tidak diayak yang dilembapkan dengan medium Mandel asas (pH 8) kepada nisbah 1:2.5 (pepejal:kelembapan) pada suhu 35 °C untuk 9 hari menghasilkan 1.2–49.4 kali ganda lebih tinggi aktiviti enzim lignoselulolitik berbanding dengan eksperimen kawalan. Endoglukanase, β-glukosidase dan xilanase boleh dikategorikan sebagai enzim stabil haba sederhana dan mereka juga lebih stabil dalam pH berasid. Manakala, ligninase mempunyai ciri-ciri stabil haba dan stabil alkali. Kultur bersama menghasilkan 1.9–11.8 kali ganda lebih tinggi gula terturun daripada yang dihasilkan oleh kultur tunggal dalam proses penguraian berenzim hampas kayu. Penggunaan enzim kultur bersama juga menghasilkan 3.6–85.4% lebih tinggi gula terturun dan juga 1.3–2.3 kali ganda kepekatan rafinosa, selobiosa, maltosa, glukosa dan xilosa berbanding dengan menggunakan larutan enzim selulase komersial (Celluclast). Kesimpulannya, kajian ini telah menghasilkan mikrob kultur bersama yang boleh digunakan untuk meningkatkan penghasilan enzim lignoselulolitik dan gula terturun menggunakan hampas kayu yang tidak dirawat sebagai substrat.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF	-	Acid Detergent Fibre
BSA	-	Bovine Serum Albumin
CMC	-	Carboxymethyl cellulose
DNS	-	Dinitrosalicyclic acid
EPS	-	Exopolysaccharides
FTIR	-	Fourier Transform Infrared Spectroscopy
g	-	Gram
h	-	Hour
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen Peroxides
HPLC	-	High Performance Liquid Chromatography
kDa	-	Kilo Dalton
L	-	Liter
LiP	-	Lignin peroxidase
min	-	Minute
mL	-	Milliliter
mm	-	Millimeter
MnP	-	Manganese peroxidase
MW	-	Molecular Weight
NaOH	-	Sodium hydroxide
NA	-	Not available
NAG	-	N-Acetyl-ᴅ-Glucosamine
NDF	-	Neutral Detergent Fibre

nm	-	Nanometer
°C	-	Degree Celsius
PAGE	-	Polyacrylamide Gel Electrophoresis
PDA	-	Potato Dextrose Agar
pNPG	-	p-nitrophenyl β-D-glucoside
RID	-	Refractive Index Detector
rpm	-	Rotation per minute
SEM	-	Scanning Electron Microscopy
SDS	-	Sodium Dodecyl Sulfate
SmF	-	Submerged Fermentation
SSF	-	Solid-State Fermentation
U/g	-	Unit of enzyme per gram
U//Lh	-	Unit per litre per hour
v/v	-	Volume per volume
w/v	-	Weight per volume
μL	-	Micro liter
μm	-	Micro meter

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Research

Wood-based industry in Malaysia began in the early 1900s (Ramasamy *et al.*, 2015). Starting with only to meet the domestic demand at the time, wood-based activities in Malaysia such as logging, sawmilling, primary and secondary manufacturing, have played an important role in the economic development of the country, in which they contributed 2% of the Malaysian Gross Domestic Product (GDP) and 2.7% of the country's total merchandise exports (Malaysian Timber Council, 2014). In year 2014, Malaysia has produced 3,218,515 m^3 of logs, 1,893,949 m^3 of sawn timber and 3,099,371 m^3 of plywood, with Japan, USA and India are the top three leading export destinations for these local timber products. The export of Malaysian wood-based products has recorded a positive growth of 5.1% with total exports of RM 20.5 billion (Malaysian Timber Council, 2014). Wooden furniture remained as the biggest export item contributing RM 6.3 billion, followed by plywood (RM 5.2 billion), sawntimber (RM 2.5 billion), logs (RM 2.1 billion) and Builders' Carpentry and Joinery (BCJ) (RM 1 billion). While these wood-based industries generate profits, they also yielded a huge amount of wood wastes, which can potentially give rise to environmentally sensitive disposal issues. The issues are particularly obvious in sawmills where most of the manufacturing technology in used is old and obsolete (Tye *et al.*, 2011).

It was reported that the generation of wood wastes in the sawmilling sector of Peninsular Malaysia was approximately 45 to 50% of the total volume of saw-log input (Ramasamy *et al.*, 2015). The production of the wood waste can be found in the form of off-cuts, slabs, shavings, bark and sawdust (Mekhilef *et al.*, 2011). As one of the most common residues found in wood-manufacturing entities, sawdust is largely produced during the process of planning mills, moulding plants and furniture manufacturing (Rafiqul and Sakinah, 2012). In practise, the residues are left accumulated or discarded in landfill areas, causing environmental pollution through the generation of dust and dirt. Moreover, dumping sawdust to landfills involves additional cost due to its handling and transportation, which is another burden for the industries. Burning had also been applied as one of the economical method to dispose sawdust. However, the high sulphur content of wood may result in the formation of sulphur dioxide during incineration, thereby aggravating air pollution and decreasing air quality in the vicinity (Buraimoh *et al.*, 2015). In view of these issues, research on the utilization of sawdust to turn into value-added products is of high interest as a way to manage wood residues, especially in the country like Malaysia which has a total of 3975 wood-based manufacturing entities operating within the country (Ramasamy *et al.*, 2015).

Sawdust had been used as a raw material in the derivation of biochar (Ghani *et al.*, 2013), commercial mineral-bonded cement composites (Frybort *et al.*, 2008) and as bulking agent in the composting systems (Zhou *et al.*, 2014). The utilization of sawdust also includes as a source of fuel for the cyclone gasification system (Miskam *et al.*, 2009) and for energy generation in the boilers (Ramasamy *et al.*, 2015). Sawdust gained another credit in biomass research area for being classified as lignocellulosic material with significant proportion of cellulose, hemicellulose and lignin constituted in its chemical composition. On a dry basis, sawdust contains cellulose (31.99%), hemicellulose (13.33%) and lignin (44.36%) with the rest consisting of extractives and ash (Belewu, 2006). Several potential value-added products could be derived from biodegradation of these lignocellulose components. Degradation of cellulose and hemicellulose polymers could produce hexose or pentose sugars which served as important raw material for ethanol production, while lignin degradation has huge potential for the synthesis of a number of useful chemicals such as vanillin, phenol, quinone and acetic acids (Hamid *et al.*, 2014). Biodegradation of cellulose, hemicellulose and lignin from lignocellulosic residue is very much associated to the efficiencies of lignocellulolytic enzymes to degrade the lignocellulose components (Sánchez, 2009). The effectiveness of the enzymatic mixture is highly dependent on their specific functionality to degrade specific type of lignocellulosic material. The use of same material for enzymes production and degradation was suggested to produce enzymes composition that might be then tailored for degradation functionality of that specific material (Pensupa *et al.*, 2013). It is therefore logical and necessary to produce on-site, tailor-made lignocellulolytic enzymes that are optimized for biodegradation of specific lignocellulosics material.

Due to their degrading capabilities, lignocellulolytic enzymes find application in various type of industrial field such as textile, detergent, food, animal feed, pulp and paper (Niladevi, 2007; Singh *et al.*, 2007). The field of industrial enzymes production represents the heart of biotechnology. It was estimated that the global market for commercial enzymes reached $3.3 billion in 2010, with the annual growth rate of 6% over 5-year forecast period (Thomas *et al.*, 2013). One of the major issues faced by the global enzymes manufacturing companies is the high cost of raw material, which contributes 40–60% of the total production cost (Singhania *et al.*, 2010). Therefore, efforts were made to reduce the cost of production by using cheaper and abundantly available substrates to produce enzymes with high activity (Alam *et al.*, 2009a; Jabasingh and Nachiyar, 2011; Bansal *et al.*, 2012; Yoon *et al.*, 2014). At present, there are limited studies that describe the utilization of sawdust as a substrate for the production of lignocellulolytic enzymes (Liu *et al.*, 2006; Poorna and Prema, 2007; Bansal *et al.*, 2012). The sawdust was either used as the minor substrate or been chemically pretreated prior to fermentations. None have focused on the use of untreated sawdust as a sole substrate for enzymes production. The use of untreated substrate is preferred because additional pre-treatment process with either acidic or alkaline solvents may eventually produce by-products such as furfural, 5-hydroxymethyl-2 furfural, acetic acid, phenols, heavy metals, levulinic acids and formic acids, with inhibitory effects to the microbial growth and respiration (Ang *et al.*, 2013).

Lignocellulosic materials are fermented by lignocellulolytic microorganisms in the process to produce lignocellulolytic enzymes. The fermentation can be conducted via two different fermentation approaches, submerged fermentation or solid state fermentation (Hansen *et al.*, 2015). Submerged fermentation (SmF) has been the most popular and conventional fermentation technology used by enzymes manufacturing companies such as Novozymes and Genencor (Singhania *et al.*, 2010). However, in nature, the growth and lignocellulose utilization of microorganisms secreting lignocellulolytic enzymes are more closely resemble solid-state fermentation (SSF) condition than the presence of excess water provided by SmF (Hansen *et al.*, 2015). SSF is the fermentation method that is carried out without apparent presence of water, but with sufficient moisture to support the growth of microorganisms on the solid matrix (Pandey, 2003). One of the most added advantages offered by SSF is that enzymes titers are higher than those obtained from SmF (Couto and Sanromán, 2005). This advantage has been associated with a larger biomass and lower product breakdown as observed in SSF process (Viniegra-González *et al.*, 2003). In addition, energy expenditure is lower for SSF compared to SmF since less water is needed, no mechanical mixing and less energy requirement in downstream processing (Hansen *et al.*, 2015). Furthermore, higher concentration of products can be obtained from SSF, making purification works such as concentration and freeze drying are undesirable (Zhuang *et al.*, 2007).

The lignocellulolytic enzymes production also depends on the type of microbial strain and the strains giving higher activities on lignocellulosic material in SSF condition are important. *Aspergillus*, *Trichoderma*, *Rhizopus*, *Fusarium* and *Penicillium* are some of the fungi genera reported able to produce lignocellulolytic enzymes in SSF (Hansen *et al.*, 2015). For bacteria, *Bacillus* and *Streptomyces* are the most common been reported (Krishna, 1999; Niladevi *et al.*, 2007). The fungal and bacterial strains were isolated from substrata containing lignocellulosic carbon source such as residues from different agricultural sectors, soil and debris from cereal production (Jabasingh and Nachiyar, 2011; Irfan *et al.*, 2012; Ang *et al.*, 2013). Another interesting source to prospect for lignocellulolytic microorganisms is from the guts of insects. Some insects relied upon their gut microbial community to degrade lignocellulosic material as their nutrient sources. One of these insects is termite. Termites were reported to harbouring diverse array of lignocellulolytic microorganisms inside their gut (Dheeran *et al.*, 2012). Several lignocellulolytic microorganisms such as *Pseudomonas*, *Bacillus*, *Enterobacter*, *Streptomyces*, *Paenibacillus*, *Aspergillus* and *Sporothrix* had been successfully isolated from termite species of *Coptotermes curvignathus* (Ramin *et al.*, 2009), *Reticulitermes santonensis* (Matkar *et al.*, 2013) and *Amitermes hastatus* (Le Roes-Hill *et al.*, 2011). However, the capability of the microorganisms originated from termite's gut to produce lignocellulolytic enzymes have only been studied in culture employing SmF technique. The potential of microorganisms isolated from termite gut in producing lignocellulolytic enzymes under SSF remained to be addressed.

Earlier reports are available for the production of lignocellulolytic enzymes by single culture of bacteria and fungi from termite gut (Wenzel *et al.*, 2002; Ramin *et al.*, 2009; Le Roes-Hill *et al.*, 2011; Dheeran *et al.*, 2012). However, a single microorganism cannot produce all the enzymes necessary for complete bioconversion of lignocellulose and different microorganisms are normally co-exist symbiotically on solid substrates in nature (Yoon *et al.*, 2014). Thus, co-culturing of microorganisms which act synergistically for rapid bioconversion of lignocellulosic biomass under SSF, is attractive (Wang *et al.*, 2006; Kumar *et al.*, 2008a). Co-culture defined as inoculation of different specified microbial strains under aseptic conditions, had been used to achieve improved production of biologically active compounds such as organic acids, vitamins and antibiotics (Bader *et al.*, 2010). Similarly, co-culture is beneficial for production of lignocellulolytic enzymes during biodegradation of lignocellulosic substrate (Brijwani *et al.*, 2010; Dhillon *et al.*, 2011; Kolasa *et al.*, 2014) as they offer higher productivity of enzymes and better adaptability compared to single culture (Dashtban *et al.*, 2010). Hence, it is hypothesized that through co-culture techniques, improved level of lignocellulolytic enzymes produced by synergistic interactions between different microorganisms may be achieved in single process. It may further eliminates the requirement to cultivate multiple single cultures separately, followed by enzymes blending which then increases the cost of double equipment needed (Kolasa *et al.*, 2014). As termite gut was known to contain dense population of microbiota that work co-operatively in lignocellulosic material decomposition, co-culturing microorganisms originated from such sources remained as an interesting topic to be further explored.

1.2 Objectives

The objectives of this research are as follows:

1. To isolate, screen and identify the termite guts microorganisms with the capability to produce lignocellulolytic enzymes in SSF using untreated sawdust as substrate.
2. To construct and to evaluate the compatibility of the members in microbial co-culture with promising level of lignocellulolytic enzymes activities. The profile of lignocellulolytic enzymes produced by both single and microbial co-culture and its relation with exopolysaccharides production, N-acetyl-D-glucosamine and protein concentration will be analysed.
3. To study the effect of sawdust particle size, incubation temperature, pH and moisture content on the production of lignocellulolytic enzymes by varying one variable at a time.
4. To characterize the lignocellulolytic enzymes produced by a selected microbial co-culture in terms of its optimum pH, optimum temperature, pH stability and thermal stability. A sawdust-based biorefining strategy for reducing sugars production will be developed.

1.3 Scope of the Research

This study focused in investigating the capability of termite gut's microorganisms to produce lignocellulolytic enzymes under SSF by using untreated sawdust as solid substrate. The bacterial and fungal isolates from *Bulbitermes* sp. termite gut were screened through qualitative approach by using plate base technique and the lignocellulolytic activities were assessed quantitatively in SSF condition. Lignocellulolytic activities were calculated based on the endoglucanase, exoglucanase, β-glucosidase, xylanase, lignin peroxidase, manganese peroxidase and laccase activities. The isolates with highest lignocellulolytic activities were selected, identified and further used for the development of microbial co-culture.

The effect of sawdust particle size, incubation temperature, pH of the medium and moisture content on lignocellulolytic enzymes production were studied. An optimal condition for the enzymes production was set. Lignocellulolytic enzymes obtained from the optimal SSF condition were characterized by means of determination of their optimal temperature, pH, and stability.

The biodegradation potential by single and microbial co-culture cultivated under SSF were studied. A sawdust-based biorefining strategy was developed by extracting the lignocellulolytic enzymes produced from SSF process and then used to hydrolyse the fermented sawdust. The amounts of reducing sugars obtained after the hydrolysis process were measured.

1.4 Significance of the Research

As Malaysia has significant amount of woody-based activities such as logging and saw-milling, the mass generation of sawdust as the most common wood residues produced by the forestry related industries, can potentially give rise to environmentally sensitive disposal issues (Hoi, 2003). This had urged the need to properly utilize the sawdust to turn into various value-added products including as raw material for the production of lignocellulolytic enzymes. Below are several identified issues which make the current research is significance:

i. Improper management of wood residues including sawdust can give an adverse effect towards the air quality, which remained an issue to be solved by the parties involved including the local community, the wood-based industries themselves and the government enforcement bodies. Sawdust is therefore proposed as an alternative raw material that serves as substrate for the production of lignocellulolytic enzymes.

ii. The afore mentioned suggestion is in line with the Malaysian government effort in exploiting the country's biomass resources up to its optimum level as outlined in National Biomass Strategy 2020 (Agensi Inovasi Malaysia, 2011). From Malaysian perspectives as an important global exporter for wood-based products, the use of sawdust for lignocellulolytic enzymes production is a promising technology to add more value to the wood residues as well as providing more opportunities to achieve economic advancement for the industrial player.

iii. The cost of raw material contributes for about 40–60 % of the total enzyme production cost. A cheaper alternative substrate can be prospected as a way to reduce the production cost by using the raw untreated sawdust as sole substrate in the process of lignocellulolytic enzymes production. The lack of chemical or/and physical pre-treatment step during the substrate preparation stage could further reduce the cost of overall enzyme production. Furthermore, enzymes production in SSF can also facilitates a lower capital operating cost due to less water requirements and lower energy expenditures. This study is regarded as the first to describe the use of untreated sawdust as a sole solid support in SSF for lignocellulolytic enzymes production.

iv. Investigation to find new isolates from habitats containing lignocellulosic substrates with the capability to produce lignocellulolytic enzymes are relatively simple strategies to obtain higher titre of enzymatic activities in facilitating the biomass degradation process. Since termite gut stands as a rich source to prospect for diverse and efficient lignocellulose degrading microorganisms, the current study described the potential of termite gut microorganisms in producing lignocellulolytic enzymes and also degrading untreated sawdust under SSF condition.

v. Although several studies have described the capability of termite gut microorganisms to exhibit lignocellulolytic activities, none has reported the effect when the microorganisms are co-cultured together. As termite gut holds a dense population of microorganisms, co-culturing may provide an insight into types of interactions existed between the guts microbiota.

vi. Knowledge on self-production of lignocellulolytic enzymes is essential as tailor-made enzymatic mixtures that are optimized for the degradation of specific type of lignocellulosics remains as a strategic issue to be considered during the development of a sustainable biomass-biorefinery process. The use of same material for enzyme production and degradation process could be a way to obtain optimal degradation results of that specific material. Therefore, cultivation of microorganisms on sawdust was projected to produce lignocellulolytic enzymes with specific functionality to degrade sawdust and simultaneously promoting a greener technology as a way to manage woody residues in Malaysia.

1.5 Thesis Organization

This thesis is organized into ten chapters. **Chapter 2** covers relevant literatures on the availability of lignocellulosic biomass in Malaysia, structure of lignocellulose and the potential of sawdust to be used as raw material for production of high value products. This chapter provides an overview of lignocellulose degradation via acidic and enzymatic approach, and the role played by lignocellulolytic enzymes (cellulases, xylanase and ligninases) in the enzymatic degradation process. The source to prospect for lignocellulolytic microorganisms and the plausibility of termite gut to serve as a good reservoir for isolation of such microorganisms were explained. This chapter also deals with information on SmF and SSF as well as important SSF process variables related to the production of lignocellulolytic enzymes. The positive role of microbial co-culture in lignocellulolytic enzymes production was also reviewed. Literatures related to application of lignocellulolytic enzymes in various industries are briefly summarized.

Chapter 3 describes the general experimental procedures performed in this research. All common methods and procedures are placed in this chapter and be referred to in specific chapters, respectively.

The results and discussions are divided into six main chapters. **Chapter 4** describes the isolation, screening and identification of lignocellulolytic microorganisms from *Bulbitermes* sp. termite gut. **Chapter 5** presents the development of microbial co-culture from the selected lignocellulolytic microorganisms in order to improve the enzymatic activities. In **Chapter 6**, a thorough comparison was made between the lignocellulolytic enzymes activities produced by microbial co-culture with its respective single culture member. The profile of lignocellulolytic activities together with its relation with exopolysaccharides production, N-acetyl-D-glucosamine and protein concentration was described. **Chapter 7** provided the evaluation of the effect of SSF operating parameters on lignocellulolytic enzymes activities. **Chapter 8** presents the characteristics of lignocellulolytic enzymes produced by microbial co-culture in terms of optimal temperature and pH, temperature and pH stability. Activity staining and molecular mass of lignocellulolytic enzymes on SDS-PAGE gel were determined. The capability of single and microbial co-culture of termite gut's microorganisms to degrade lignocellulose and the development of sawdust-based biorefinery strategy were presented in **Chapter 9**.

The conclusions from this research are given in **Chapter 10**. This chapter also states specific achievements, problems and some recommendations for future work.

10.2 Recommendations

The utilization of sawdust as a raw material for the production of highly valuable lignocellulolytic enzymes and fermentable sugars will not only fetch valuable remuneration for wood-based industries, but also help mitigate environmental pollution. In addition, through the study of microorganisms and their enzymatic activities, the mechanisms of efficient lignocellulose degradation in the termite gut may then could be elucidated, findings which have significant potential in biorefinery industries. The defined microbial co-culture also stands as a useful technique to improve the titre of lignocellulolytic enzymes activities and therefore worthy of future study. Some recommendations for future studies are outlined as follows:

i. As sawdust was observed to contain high content of lignin, it is very interesting to extract the lignin through enzymatic or biological approach as this natural polymer can serve as a base for different materials application in the fields of bioplastics, (nano) composites and nanoparticles.

ii. Since termite is one of the insects with a dense population of microorganisms living symbiotically inside its guts it is highly expected that the termite gut also resides microorganism that could influence the performance of the fermentation system, including hydrogen yield. Biohydrogen is regarded as one of potentially advantageous alternative energy to minimise or even eliminate the dependability on fossil fuels. Future research should consider prospecting and characterising hydrogen-producing microorganisms from the guts of termites.

iii. To obtain the best production of enzymes, identification of optimal ratio between the two microorganisms in a co-cultivation is necessary. The addition of termite extract into the medium or substrate can also be considered as a strategy to enhance the growth of microorganisms isolated from the termite gut. It is also feasible to construct an efficient lignocellulolytic enzymes producing–co-culture for reducing sugars preparation from lignocellulosic biomass by adjusting the microbial constituent proportions in the consortium.

iv. The present study was able to show that reducing sugars can be produced from the enzymatic degradation of sawdust. Future studies should focus to investigate whether the reducing sugars can be further fermented by microorganisms to make ethanol from sawdust.

v. Static tray fermentation is often used for large-scale production of enzymes, as it offers potential benefits over bioreactors, such as simple technique, trays can be stacked over one another in shelves and higher yields. Solid-state tray fermentation could be possibly used to achieve higher yield of lignocellulolytic enzymes due to the capacity to put high substrate loading, large area for microorganism to grow and easy handling bioreactor as compared to immersion, packed-bed and rotating drum bioreactor. A more comprehensive study is needed to provide information about the production of lignocellulolytic enzymes in solid-state tray fermentation employing co-culture of selected microorganisms.

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